Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) Protein Domains Target LipY Lipases of Pathogenic Mycobacteria to the Cell Surface via the ESX-5 Pathway

Daleke, M.H.; Cascioferro, A.; de Punder, K.; Ummels, R.; Abdallah, A.M.; Wel, N.; Peters, P.J.; Luirink, S.; Manganelli, R.; Bitter, W.

published in
Journal of Biological Chemistry
2011

DOI (link to publisher)
10.1074/jbc.M110.204966

document version
Publisher's PDF, also known as Version of record

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 21, pp. 19024–19034, May 27, 2011

Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) Protein Domains Target LipY Lipases of Pathogenic Mycobacteria to the Cell Surface via the ESX-5 Pathway*[S]

Received for publication, November 23, 2010, and in revised form, April 5, 2011 Published, JBC Papers in Press, April 6, 2011, DOI 10.1074/jbc.M110.204966

Maria H. Daleke[‡§], Alessandro Cascioferro[¶], Karin de Punder[‖], Roy Ummels[‡], Abdallah M. Abdallah[‡‖], Nicole van der Wel[‖], Peter J. Peters[‖], Joen Luirink[§], Riccardo Manganelli[¶], and Wilbert Bitter[‡§1]

From the [‡]Department of Medical Microbiology and Infection Control, VU University Medical Centre, 1081 BT Amsterdam, The Netherlands, the [§]Department of Molecular Microbiology, VU University, 1081 HV Amsterdam, The Netherlands, the [¶]Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, 35121 Padua, Italy, and [‖]The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, 1066 CX Amsterdam, The Netherlands

The type VII secretion system ESX-5 is a major pathway for export of PE and PPE proteins in pathogenic mycobacteria. These mycobacteria-specific protein families are characterized by conserved N-terminal domains of 100 and 180 amino acids, which contain the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs after which they are named. Here we investigated secretion of the triacylglycerol lipase LipY, which in fast-growing mycobacteria contains a signal sequence, but in slow-growing species appears to have replaced the signal peptide with a PE or PPE domain. Selected LipY homologues were expressed in wild-type *Mycobacterium marinum* and its corresponding ESX-5 mutant, and localization of the proteins was investigated by immunoblotting and electron microscopy. Our study shows that *Mycobacterium tuberculosis* PE-LipY ($LipY_{tub}$) and *M. marinum* PPE-LipY ($LipY_{mar}$) are both secreted to the bacterial surface in an ESX-5-dependent fashion. After transport, the PE/PPE domains are removed by proteolytic cleavage. In contrast, *Mycobacterium gilvum* LipY, which has a signal sequence, is not transported to the cell surface. Furthermore, we show that $LipY_{tub}$ and $LipY_{mar}$ require their respective PE and PPE domains for ESX-5-dependent secretion. The role of the PE domain in ESX-5 secretion was confirmed in a whole cell lipase assay, in which wild-type bacteria expressing full-length $LipY_{tub}$, but not $LipY_{tub}$ lacking its PE domain, were shown to hydrolyze extracellular lipids. In conclusion, both PE and PPE domains contain a signal required for secretion of LipY by the ESX-5 system, and these domains are proteolytically removed upon translocation.

Mycobacteria such as *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, have a highly unusual and complex cell envelope (1). To secrete virulence factors across the cell envelope, these bacteria use specialized protein secretion systems, known as ESX or type VII secretion systems (2–8). Mycobacterial genomes contain up to five genetic loci coding for type VII secretion systems, named ESX-1 to ESX-5 (9, 10). The most well studied system, ESX-1, is responsible for the secretion of 10 substrates, including the important T-cell antigens ESAT-6 and CFP-10, and is required for full virulence of *M. tuberculosis* (7, 11–17). Phylogenetic analyses and comparative genomics suggest that the five ESX clusters have evolved by gene duplication and that ESX-5 is the result of the most recent duplication event (9). Interestingly, ESX-5 is restricted to a group of mycobacterial species known as the slow-growing mycobacteria, which include all major pathogens, such as *M. tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium ulcerans*, and the fish pathogen *Mycobacterium marinum* (9). Four of the ESX loci contain also PE and PPE genes (named after the conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs near the N termini of their respective gene products) (9, 10), and the appearance of ESX-5 predates the huge expansion of these gene families in slow-growing mycobacteria (18). Intriguingly, although fast-growing, non-pathogenic mycobacteria encode only a small number of PE and PPE proteins, pathogens such as *M. marinum* and *M. tuberculosis* dedicate nearly 10% of the coding potential of their genomes to members of the PE and PPE gene families (19). Although the precise function of these proteins is largely unknown, members of both families are important for mycobacterial virulence (20–24). Moreover, various PE and PPE proteins are located on the cell surface, where they can interact with the host during infection (21, 25–28). However, because the PE and PPE proteins lack detectable secretion signals, the route of translocation across the cell envelope remained unclear until members of the two protein families were recently shown to be secreted by the ESX-5 system in *M. marinum* (3, 29).

PE and PPE proteins are characterized by unrelated, conserved N-terminal domains[2] of ~100 (PE) and ~180 (PPE)

* This work was supported by funding from an "Earth and Life Sciences (ALW) Open Program" grant from the Netherlands Organization for Scientific Research (NWO) (to M. H. D.) and from the European Community Seventh Framework Programme (FP7/2007-2013) under Grant Agreement 201762.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.
[1] To whom correspondence should be addressed: van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Fax: 31-20-4448318; E-mail: w.bitter@vumc.nl.

[2] The abbreviations used are: PE/PPE domains, conserved 100 aa (PE) and 180 aa (PPE) N-terminal domains that contain the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs after which the mycobacteria-unique PE/PPE proteins are named; aa, amino acids; TAG, triacylglycerol; PE_PGRS, polymorphic GC-rich repetitive sequence subfamily of PE proteins; BCG, bacille Calmette-Guérin; Tn, transposon; OD, optical density; PPE_MPTR, major polymorphic tandem repeat subfamily of PPE proteins.

amino acids (aa) (19), and they can be divided into different subfamilies on the basis of their C-terminal domains (19, 30, 31). To date, little is known about the function of the various domains of the PE and PPE proteins. In fact, the *M. tuberculosis* PE protein LipY (hereafter referred to as $LipY_{tub}$) is the only PE protein for which a function has been characterized (32). *M. tuberculosis* $LipY_{tub}$ is involved in the degradation of triacylglycerols (TAGs) and is the major active lipase under nutrient-deprived conditions. This led to the hypothesis that $LipY_{tub}$ plays a role in fatty acid metabolism during the dormancy and reactivation stages of the *M. tuberculosis* infection cycle (32). Although the lipolytic activity of $LipY_{tub}$ is expressed by the C-terminal part of the protein (32), the function of its N-terminal PE domain is less clear. It has been shown that the PE domain of $LipY_{tub}$ has an inhibiting effect on the lipase activity (33). Furthermore, cell wall and cell surface localization of LipY was shown to occur independently of the presence of the PE domain (33). Similar results were obtained for *M. marinum* LipY (hereafter referred to as $LipY_{mar}$), which contains a PPE domain instead of a PE domain. These results contradict those obtained in Refs. 26 and 27, which showed that localization of heterologously expressed PE_PGRS33 (member of the polymorphic GC-rich repetitive sequence subfamily of PE proteins) in the cell wall of *Mycobacterium smegmatis* is dependent on the PE domain.

In this study, we show that, like many other PE and PPE proteins, the *M. tuberculosis* and *M. marinum* LipY homologues are secreted by ESX-5. Using a combination of molecular techniques and analysis of the lipolytic activity of LipY, we show that ESX-5-mediated secretion of the LipY homologues is dependent on the respective PE and PPE domains.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*M. marinum* wild-type strains E11 (34) and M^{vu} (35) and their respective ESX-5 mutants 7C1 (29) and Mx2 (3) and *Mycobacterium gilvum* PYR-CGK (ATCC 700033) (36) were grown at 30 °C with shaking at 90 rpm, whereas *Mycobacterium bovis* bacille Calmette-Guérin (BCG) Copenhagen (37), *M. bovis* BCG Tice (38), and *M. tuberculosis* mc^26020 (39) were grown standing at 37 °C. *M. smegmatis* mc^2155 (40) was grown at 37 °C with shaking at 90 rpm. All mycobacterial strains were grown in Middlebrook 7H9 broth (Difco-BD Biosciences), supplemented with Middlebrook ADC and 0.05% Tween 80 (Sigma-Aldrich). In secretion experiments, mycobacteria were grown to mid-logarithmic phase, at which they were washed to remove BSA (part of the ADC supplement) and subsequently grown in Middlebrook 7H9 supplemented with 0.2% dextrose and 0.05% Tween 80 for another 24 h. If cultures were grown for electroporation, glycine was added to a final concentration of 2.5 mg/ml during the early exponential phase to increase electroporation efficiency (3). Transformants were selected on Middlebrook 7H10 agar plates and supplemented with Middlebrook OADC and appropriate antibiotics. *Escherichia coli* DH5α was grown at 37 °C in LB medium (Difco-BD Biosciences) for cloning experiments. Hygromycin was used at a concentration of 50 μg/ml for mycobacteria and 100 μg/ml for *E. coli*, and kanamycin was used at a concentration of 25 μg/ml.

Plasmid Construction—All DNA manipulations were carried out according to standard protocols. *Pfu* DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Fermentas. Chromosomal DNA from *M. marinum* E11 and *M. gilvum* PYR-CGK was used as template in PCR reactions. Plasmids and primers are described in supplemental Tables S1 and S2, respectively.

Cloning of *Rv3097c* (gene encoding $LipY_{tub}$), modified to express a C-terminal HA tag, in the *E. coli*-mycobacterial shuttle vector pMV10–25 to generate pAL36 is described in Ref. 26. pMV10–25 contains a minimal pAL5000 origin of replication, which lacks *orf5* that is required for initiating stable replication (41). Therefore, a 1749-bp fragment containing the *hsp60* promoter sequence, the *Rv3097c* gene, and the in-frame sequence encoding an HA tag followed by a stop codon was isolated from pAL36 by digestion with HindIII and XbaI. The fragment was ligated into the pSMT3 vector (42), which contains a full pAL5000 origin of replication, digested with HindIII and XbaI, to generate pSMT3::$LipY_{tub}$. To construct pSMT3::$LipY_{tub}$-ΔPE, a gene fragment encoding $LipY_{tub}$ without the first 125 aa was amplified from pAL36, cloned in pMV10–25 to generate pAL45, and subsequently transferred to pSMT3 by digestion with HindIII and XbaI.

For cloning of sequences encoding $LipY_{mar}$ and $LipY_{gilv}$ or truncated forms of $LipY_{tub}$ and $LipY_{mar}$, forward primers were designed to contain an NheI site immediately upstream of the start codon, and reverse primers contained an HA tag encoding sequence in-frame with the coding sequence, a stop codon, and a BamHI or BglII site. PCR fragments were inserted into pSMT3::$LipY_{tub}$, digested with NheI and BamHI, thereby replacing the *Rv3097c* gene and HA-encoding sequence.

Plasmids for expression of fusions of $LipY_{tub}$ and $LipY_{gilv}$ were generated as follows. To replace the PE domain of $LipY_{tub}$ with the signal peptide of $LipY_{gilv}$, a 145-bp fragment encoding the signal sequence was amplified from *M. gilvum* chromosomal DNA and digested with NheI and EcoRI. Then, a 1045-bp fragment coding for the linker and lipase domains of $LipY_{tub}$ and the HA tag was isolated from pSMT3::$LipY_{tub}$ by digestion with EcoRI and XbaI. The two fragments were ligated into pSMT3, digested with NheI and XbaI, to generate pSMT3::SP_{gilv}-$LipY_{tub}$. For the construct in which the signal peptide of $LipY_{gilv}$ was replaced by the PE domain of $LipY_{tub}$, a 309-bp fragment encoding the $LipY_{tub}$ PE domain was isolated from pSMT3::$LipY_{tub}$ by digestion with NheI and EcoRI. In addition, a 1390-bp fragment encoding the linker and lipase domains of $LipY_{gilv}$ and the HA sequence was amplified from *M. gilvum* chromosomal DNA and digested with EcoRI and BglII. These two fragments were ligated into a 5691-bp NheI-BamHI fragment of pSMT3::$LipY_{tub}$, replacing the *Rv3097c* gene, resulting in the pSMT3::PE_{tub}-$LipY_{gilv}$ vector. For the fusion of the PE and linker domains of $LipY_{tub}$ to the lipase domain of $LipY_{gilv}$, a nested PCR approach was taken to amplify fragments from the pSMT3::$LipY_{tub}$ and pSMT3::$LipY_{gilv}$ vectors. After digestion with NheI and BamHI, the resulting 1436-bp fragment was inserted into pSMT3::$LipY_{tub}$, as described above, to generate pSMT3::$PE/linker_{tub}$-$LipY_{gilv}$.

Because *M. tuberculosis* mc^26020 carries a hygromycin resistance marker (39), $LipY_{tub}$ was placed under control of the

hsp60 promoter in the pBH10 vector (3, 43). A 1355-bp fragment containing *Rv3097c*, the in-frame HA sequence, and the stop codon was cut out from pSMT3::$LipY_{tub}$ with BspEI and BamHI, and ligated into pBH10 digested with the same enzymes. The constructs were verified by sequencing.

Site-directed Mutagenesis of the $LipY_{tub}$ Cleavage Site—Derivatives of pSMT3::$LipY_{tub}$ containing point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Because pSMT3::$LipY_{tub}$ was too large for the *Pfu* Turbo DNA polymerase to amplify, the smaller pAL36 was used as template. The 1749-bp HindIII-XbaI fragments containing the *hsp60* promoter and the mutated gene were transferred into pSMT3 as described above. The mutations were verified by sequencing.

SDS-PAGE and Immunoblotting—Mycobacterial cells grown to mid-logarithmic phase were separated from culture supernatants by centrifugation. The cells were washed with PBS and thereafter disrupted by sonication. Surface-exposed proteins were isolated by incubating intact cells with 0.5% Genapol X-080 (v/v; Sigma-Aldrich) for 30 min at room temperature with head-over-head rotation, prior to sonication (26). Secreted proteins were precipitated from culture supernatants with 10% TCA (w/v; Sigma-Aldrich). Protein samples were boiled, separated by SDS-PAGE, and thereafter transferred to nitrocellulose membranes (Amersham Biosciences) by Western blotting. The membranes were incubated with mouse monoclonal antibodies directed against the influenza hemagglutinin epitope (HA.11; Covance), GroEL2 (CS44; John Belisle, National Institutes of Health, Bethesda, MD, Contract AI-75320) or ESAT-6 (Hyb 76-8; Statens Serum Institut, Copenhagen, Denmark) or with rabbit polyclonal serum recognizing PPE41 (3). Secondary horseradish peroxidase conjugated goat anti-mouse IgGs (A106PS, American Qualex) or goat anti-rabbit IgGs (611-1302, Rockland) were detected with 4-chloronaphtol/3,3-diaminobenzidine staining or ECL (Pierce).

N-terminal Sequencing—To identify the $LipY_{tub}$ processing site, we determined the N-terminal amino acid sequence of the cleaved form of the protein. Surface-exposed proteins were extracted with Genapol as described above and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) and stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 1% acetic acid, 40% methanol. After destaining with 50% methanol, the band corresponding to processed $LipY_{tub}$ was cut out from the membrane, and the sequence of the first seven amino acids was determined by Edman degradation (Eurosequence, Groningen, The Netherlands).

Immunogold Electron Microscopy (EM)—Fixation of bacterial cells and immunogold labeling of whole cells were done as described in Ref. 17. The HA.11 mouse monoclonal (Covance) was used to visualize the HA-tagged LipY homologues.

Lipase Activity Assay—The lipolytic activity of surface-exposed $LipY_{tub}$ was analyzed with a modified method of the assay described in Ref. 33. Intact *M. marinum* cells grown in the dark (to prevent production of yellow pigment that might interfere with the absorbance measurements) were centrifuged, and an equivalent of 1 OD unit was resuspended in Tris-HCl buffer (100 mM, pH 8.0). *p*-Nitrophenyl stearate was added from a 20 mM stock solution prepared in isopropyl alcohol to reach a final concentration of 0.5 mM in 200 μl, and the mixture was vortexed and incubated for 15 min at 37 °C. Thereafter the bacteria were centrifuged, and the supernatant was transferred to a 96-well plate before measuring the production of *p*-nitrophenol with a spectrophotometer at 405 nm. Reactions were carried out in triplicate.

Bioinformatic Analyses—Nucleotide and protein sequences were retrieved from the MarinoList, TubercuList, and National Center for Biotechnology Information (NCBI) databases. Linear genomic comparisons were performed with the Artemis Comparison Tool software release 9 (44), with a cutoff value of 100 bp.

Cells Lines and Culture Conditions—For LipY macrophage infection experiments, the human acute pro-monocytic leukemia THP-1 cell line was used. Cells were cultured in RPMI 1640 with Glutamax-1 medium (Invitrogen) supplemented with 10% FCS, streptomycin, and penicillin and differentiated into macrophage-like cells in the presence of 10 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich).

LipY Processing by Mycobacteria in Macrophages—For infection, THP-1 cells were used between passage 13 and 20. Cells were seeded at 10×10^6 per flask in 75-cm^2 diameter flasks. Mononuclear cells ($3–5 \times 10^7$) were infected with *M. tuberculosis* or *M. bovis* BCG (multiplicity of infection 10) and incubated for 3 h at 37 °C and 5% CO_2. The supernatant was removed, and the infected cells were washed three times with medium to remove extracellular bacteria. Subsequently, the cells were incubated in fresh medium with 10% FCS at 37 °C and 5% CO_2 for the indicated time periods. Enrichment of mycobacterial proteins was performed as follows. Briefly, infected THP-1 cells were collected and washed twice with PBS. Cellular membranes were solubilized by treatment with 1% Triton X-100 for 15 min at room temperature. Intact mycobacteria were sedimented at $25,000 \times g$ for 30 min and subsequently disrupted by sonication. Proteins were separated by SDS-PAGE and visualized by immunoblotting using the HA.11 mouse monoclonal (Covance).

RESULTS

Homologues of LipY Have Evolved Different N-terminal Domains—M. tuberculosis LipY consists of three domains: a typical PE domain at the N terminus followed by a linker domain of unknown function and a C-terminal domain containing the triacylglycerol lipase motif (for a schematic view, see Fig. 1*A*). Nearly identical orthologues of $LipY_{tub}$ are found in various other species of the *M. tuberculosis* complex, such as *M. bovis*. However, with the exception of a pseudogene in *M. leprae* (32), only one orthologue has been reported in a mycobacterial species outside the *M. tuberculosis* complex (33). This gene is present in *M. marinum* and codes for a protein with a different domain organization. Remarkably, although the linker and lipase domains of $LipY_{mar}$ are 65% identical to $LipY_{tub}$, the N-terminal PE domain is replaced by a PPE domain (Fig. 1*A*).

We performed a BLAST search to identify additional homologues of LipY in mycobacterial species. Two *lipY* copies were found in *Mycobacterium kansasii*. Interestingly, one of these

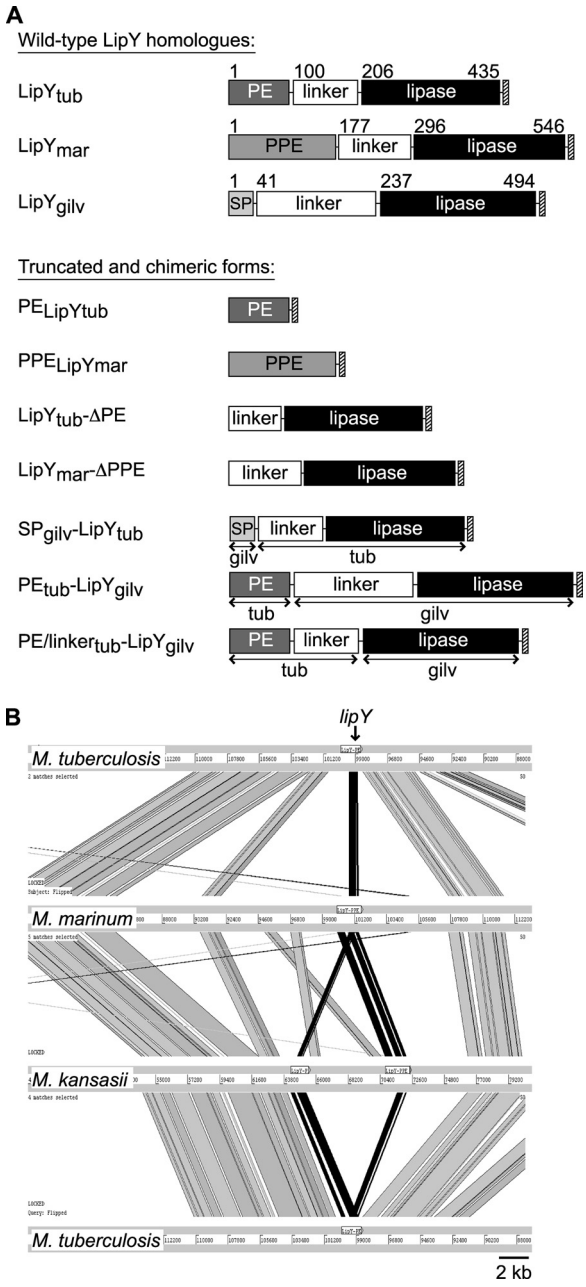

FIGURE 1. **Mycobacterial LipY homologues have evolved different N-terminal domains.** *A*, schematic representation of wild-type, truncated, and chimeric forms of *M. tuberculosis* LipY ($LipY_{tub}$), *M. marinum* LipY ($LipY_{mar}$), and *M. gilvum* LipY ($LipY_{gilv}$). The PE, PPE, and signal peptide (*SP*) domains are colored in shades of *gray*, the linker regions are *colorless*, and the lipase domains are *black*. The HA tag is depicted as a *striped box*. Domain boundaries are indicated for the wt proteins. *B*, comparisons of 30-kb regions surrounding the *lipY* genes, using the Artemis Comparison Tool, in the genomes of *M. tuberculosis*, *M. marinum*, and *M. kansasii*. Conserved regions are shown in *gray*, whereas homology within the *lipY* genes is represented in *black*. The cut-off value for sequence homology was set to 100 bp.

has a PE domain (NCBI accession number ZP_04747303), whereas the other has a PPE domain (NCBI accession number ZP_04747310). The latter variant is probably not functional because its lipase domain is disrupted by a stop codon so that this PPE-LipY protein lacks the histidine residue of the Ser-Asp-His catalytic triad. The two *M. kansasii lipY* genes are located six genes apart in a region of conserved synteny with the sequences surrounding the *lipY* genes in *M. tuberculosis* and *M. marinum* (Fig. 1*B*). Short stretches of non-homologous DNA immediately up- and downstream of the *lipY* genes indicate that some insertions and/or deletions have occurred in this region. When the BLAST analysis was extended to the fast-growing mycobacteria, two additional putative LipY proteins were identified in *M. gilvum* (hereafter referred to as $LipY_{gilv}$) and *Mycobacterium vanbaalenii* that shared 44 and 43% identity, respectively, with the C-terminal half of the linker domain and the lipase domain of $LipY_{tub}$ (NCBI accession numbers YP_001135683 and YP_952650). Strikingly, the N termini of these sequences do not share homology with PE or PPE domains but instead appear to contain classical signal peptides (Fig. 1*A*), as indicated by SignalP analysis.

To gain insight in the evolutionary relationships between the LipY homologues, a phylogenetic tree rooted to the *Nocardia farcinica* lipase Nfa23150 (NCBI accession number YP_118526) was constructed based on the conserved part of the lipase domains, using the MEGA4 software (45) (not shown). The tree topology is similar to that of the mycobacterial species tree, suggesting that the original *lipY* gene was modified to contain PE/PPE domains. The alignment tree also suggests that PE-LipY evolved before PPE-LipY. This implies that the signal sequence was initially replaced by a PE domain. Subsequently, PE-LipY was (partially) duplicated and modified to contain an N-terminal PPE domain.

$LipY_{tub}$ and $LipY_{mar}$ Are Substrates of ESX-5 in M. marinum—Because several PE and PPE proteins have been shown to be secreted by the ESX-5 secretion system (3, 29), we hypothesized that the LipY homologues with PE/PPE domains are also ESX-5 substrates. To investigate this, the genes encoding $LipY_{tub}$ and $LipY_{mar}$ were each cloned under control of the *hsp60* promoter and modified to express a C-terminal HA tag (Fig. 1*A*). The two constructs were subsequently introduced into wild-type *M. marinum* strain E11 and its ESX-5 mutant derivative 7C1, and the localization of the LipY-HA fusions was analyzed. Surface-exposed proteins were extracted by treating intact cells with the mild detergent Genapol X-080 (17, 26). For the wild-type strain, a minor fraction of both $LipY_{tub}$ and $LipY_{mar}$ was detected in the culture supernatant (Fig. 2, *A* and *B*, *lane 4*), whereas substantial amounts of both proteins were detected in the surface extracts (Fig. 2, *A* and *B*, *lane 3*). GroEL2 was absent from the surface extracts and culture supernatants, confirming the integrity of the cells. Furthermore, ESAT-6 was included in the analysis as a positive control for secretion, and this protein was normally secreted in all conditions. $LipY_{tub}$ and $LipY_{mar}$ were undetectable in the surface extracts and culture supernatants of the ESX-5 mutant (Fig. 2, *A* and *B*, *lanes 7* and *8*), which confirms our hypothesis that these two proteins are substrates of ESX-5. In addition, the LipY protein levels were markedly lower in the ESX-5 mutant as compared with in the wild-type lysates. Similar results have previously been obtained for the ESX-5 substrates PPE41 and PE_PGRS (3, 29) and indicate that when secretion is blocked, stability of the ESX-5 substrates is affected. The ESX-5-dependent secretion and surface localization of $LipY_{tub}$ was confirmed in *M. marinum* strain M^{vu} and its ESX-5 mutant Mx2 (not shown).

In the total cell lysate of the wild-type strain expressing $LipY_{tub}$, two bands are detected (Fig. 2*A*, *lane 1*). The upper 40-kDa

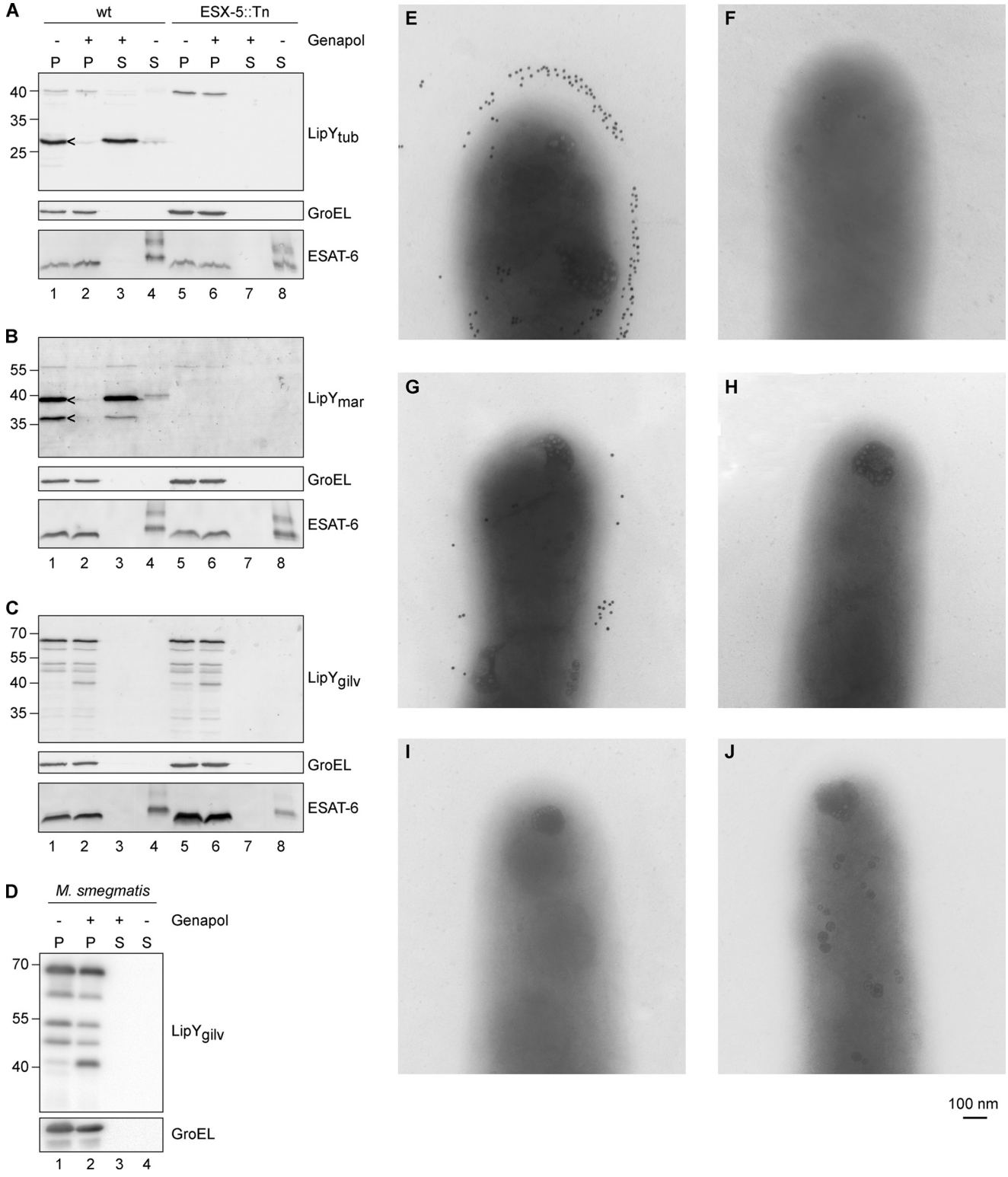

FIGURE 2. **LipY$_{tub}$ and LipY$_{mar}$ but not LipY$_{gilv}$ are secreted by ESX-5.** *A–D*, immunoblot analysis of fractions containing equivalent OD units of cell pellets (*P* −), cells treated with the mild detergent Genapol (*P* +), proteins extracted from the bacterial surface with Genapol (*S* +), and 2-fold more OD units of culture supernatants (*S* −) from *M. marinum* wild-type E11 (*wt*) and ESX-5 mutant 7C1 (*ESX-5::Tn*) (*A–C*), or *M. smegmatis* strain mc^{2}155 (*D*), expressing LipY$_{tub}$ (*A*), LipY$_{mar}$ (*B*), and LipY$_{gilv}$ (*C* and *D*). LipY is detected with an antibody recognizing the HA epitope. GroEL2 is used as control for cell lysis, and ESAT-6 is used as control for protein secretion. Processed forms of LipY$_{tub}$ and LipY$_{mar}$ are marked with *arrowheads*. *E–J*, whole bacteria fixed and probed with HA antiserum followed by goat anti-mouse conjugate labeled with 10-nm gold particles, analyzed with EM to detect surface-exposed LipY$_{tub}$ (*E* and *F*), LipY$_{mar}$ (*G* and *H*), and LipY$_{gilv}$ (*I* and *J*) in *M. marinum* wt E11 (*E*, *G*, and *I*) and the ESX-5 mutant 7C1 (*F*, *H*, and *J*). The *scale bar* represents 100 nm.

band likely represents the full-length protein, which has a calculated molecular mass of 45.9 kDa, whereas the lower band at ~28 kDa probably represents an N-terminally processed or degraded form. Only the smaller form is detectable in the surface extract and in the culture supernatant (Fig. 2*A*, *lanes 3* and *4*), whereas full-length LipY$_{tub}$ remains in the

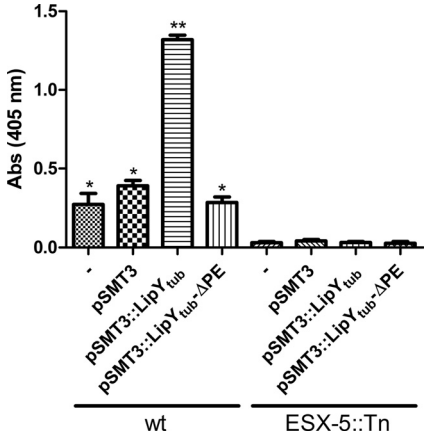

FIGURE 3. **Lipolytic activity of cell surface-exposed LipY$_{tub}$ in *M. marinum*.** Lipid-hydrolyzing activity was determined by measuring the release of *p*-nitrophenol upon incubation of intact cells of *M. marinum* wild-type E11 and its corresponding ESX-5 mutant 7C1 (*ESX-5::Tn*) overexpressing LipY$_{tub}$ and LipY$_{tub}$-ΔPE, with *p*-nitrophenyl stearate. E11 and 7C1 without plasmids or carrying the empty vector pSMT3 were included as control strains, so increases in activity can be specifically attributed to lipase exposed on the bacterial cell surface. The *bars* indicate means, and the *error bars* indicate standard deviations of two experiments, both performed in triplicate. *Abs*, absorbance.

Genapol-treated pellet and thus appears to be intracellular (Fig. 2*A*, *lane 2*). In contrast, LipY$_{tub}$ was detected as a single, 40-kDa band in the total cell lysate of the ESX-5 mutant, indicating that processing only occurs when LipY$_{tub}$ is secreted (Fig. 2*A*, *lane 5*). For LipY$_{mar}$, three bands are detected, a minor band of 56 kDa probably representing full-length LipY$_{mar}$ and two processed or degraded forms at 39 and 36 kDa (Fig. 2*B*, *lane 1*). As for LipY$_{tub}$, only the full-length band is detected in the ESX-5 mutant strain (Fig. 2*B*, *lane 5*). However, unlike for LipY$_{tub}$, all three forms are represented in the surface extract of the wild-type strain (Fig. 2*B*, *lane 3*).

To confirm the surface exposure of LipY$_{tub}$ and LipY$_{mar}$, the localization of the proteins was analyzed by immunogold EM on whole cells. Wild-type cells expressing LipY$_{tub}$ were efficiently labeled (Fig. 2*E*), whereas the ESX-5 mutant showed little or no labeling (Fig. 2*F*), consistent with the immunoblot results (Fig. 2*A*). Wild-type *M. marinum* expressing LipY$_{mar}$ also showed surface labeling (Fig. 2*G*), although to a lesser extent than LipY$_{tub}$. Again, the ESX-5 mutant showed little or no labeling (Fig. 2*H*), confirming that LipY$_{mar}$ is also secreted by ESX-5. *M. marinum* harboring the empty plasmid showed no reaction with the HA antiserum in immunoblots or whole cell immunogold EM (not shown).

To investigate whether surface-exposed LipY$_{tub}$ is functional, bacterial cells were incubated with *p*-nitrophenyl stearate, after which the bacteria were removed by centrifugation. Lipolytic activity resulted in the release of yellow *p*-nitrophenol in the supernatants, which could be measured spectrophotometrically. The wild-type strain expressing LipY$_{tub}$ showed strongly increased activity as compared with the wild-type strain harboring the empty plasmid pSMT3, confirming that LipY$_{tub}$ is secreted to the cell surface where it is enzymatically active (Fig. 3). Interestingly, the empty wild-type strain also exhibited lipolytic activity, which was completely abolished in the ESX-5 mutant strain, with or without *lipY*-containing plasmids. This activity could be due to chromosomally encoded LipY$_{mar}$.

LipY$_{gilv}$ Is Not Secreted in M. marinum or M. smegmatis—The Sec-dependent secretion pathway in mycobacteria is, as in other bacteria, responsible for translocation of proteins with signal sequences across the inner membrane (4). Because LipY$_{gilv}$ is predicted by the SignalP program to have a signal peptide, we set out to determine its subcellular localization. To this end, we amplified and cloned the *lipY$_{gilv}$* gene with a C-terminal HA tag under control of the *hsp60* promoter (Fig. 1*A*). Upon introduction into *M. marinum* wild-type E11 and its ESX-5 mutant, no bands could be detected in the culture supernatant or Genapol extract fractions (Fig. 2*C*, *lanes 3* and *4* and *lanes 7* and *8*). In the whole cell lysate, several bands were detected of which the largest is ~65 kDa (Fig. 2*C*, *lane 1*). Because LipY$_{gilv}$ has a theoretical molecular mass of 51 kDa, this could indicate that the protein is post-translationally modified. However, because a similar band was observed upon expression of *lipY$_{gilv}$* in *E. coli* (results not shown), this hypothesis is not likely to be correct. In agreement with the results obtained by immunoblotting, no labeling was observed of whole cells in immunogold EM (Fig. 2, *I* and *J*). We attempted to repeat the experiments in *M. gilvum* to rule out that LipY$_{gilv}$ is secreted by its natural host, but despite trying a variety of experimental settings, we failed to transform *M. gilvum*. Therefore, secretion was analyzed in another fast-growing species, *i.e. M. smegmatis.* Similar to *M. marinum*, several bands were observed in the cell pellet (Fig. 2*D*, *lane 1*), but also, in this host, LipY$_{gilv}$ is neither secreted nor surface-exposed (Fig. 2*D*, *lanes 3* and *4*). In conclusion, LipY$_{gilv}$ is neither exposed on the bacterial surface nor secreted to the extracellular environment.

The PE and PPE Domains Are Required for Secretion of LipY$_{tub}$ and LipY$_{mar}$ via ESX-5—To investigate the role of the conserved PE and PPE domains in ESX-5 secretion, N-terminally truncated forms of LipY$_{tub}$ and LipY$_{mar}$ lacking the first 125 and 176 aa, respectively (Fig. 1*A*), were studied. Both mutant proteins were stably produced in the cell lysates of the wild-type and mutant strains (Fig. 4, *A* and *B*, *lanes 1* and *5*). However, the absence of bands in the Genapol extracts and culture supernatants (Fig. 4, *A* and *B*, *lanes 3* and *4* and *lanes 7* and *8*) indicated that these truncated proteins were not secreted. Detection of ESAT-6 in culture supernatant fractions confirmed that the lack of secretion was specific for the LipY homologues. To exclude the possibility that LipY$_{tub}$-ΔPE and LipY$_{mar}$-ΔPPE were still secreted but could not be recognized by the antiserum, we analyzed the lipolytic activity of *M. marinum* wild-type E11 and its ESX-5 mutant expressing LipY$_{tub}$-ΔPE. As shown in Fig. 3, E11 expressing LipY$_{tub}$-ΔPE exhibits the same lipase activity as the wild-type cells. The ESX-5 mutant expressing LipY$_{tub}$-ΔPE did not show any activity. Because LipY$_{tub}$ and LipY$_{mar}$ lacking their N-terminal PE or PPE domains have been shown to have higher lipase activity than the corresponding full-length proteins (33), these results confirm that our N-terminally truncated LipY homologues are indeed not secreted.

To test whether the signal peptide of LipY$_{gilv}$ could replace the PE domain and facilitate secretion of LipY$_{tub}$ to the cell surface, we exchanged the PE domain (1–101 aa) of LipY$_{tub}$ for

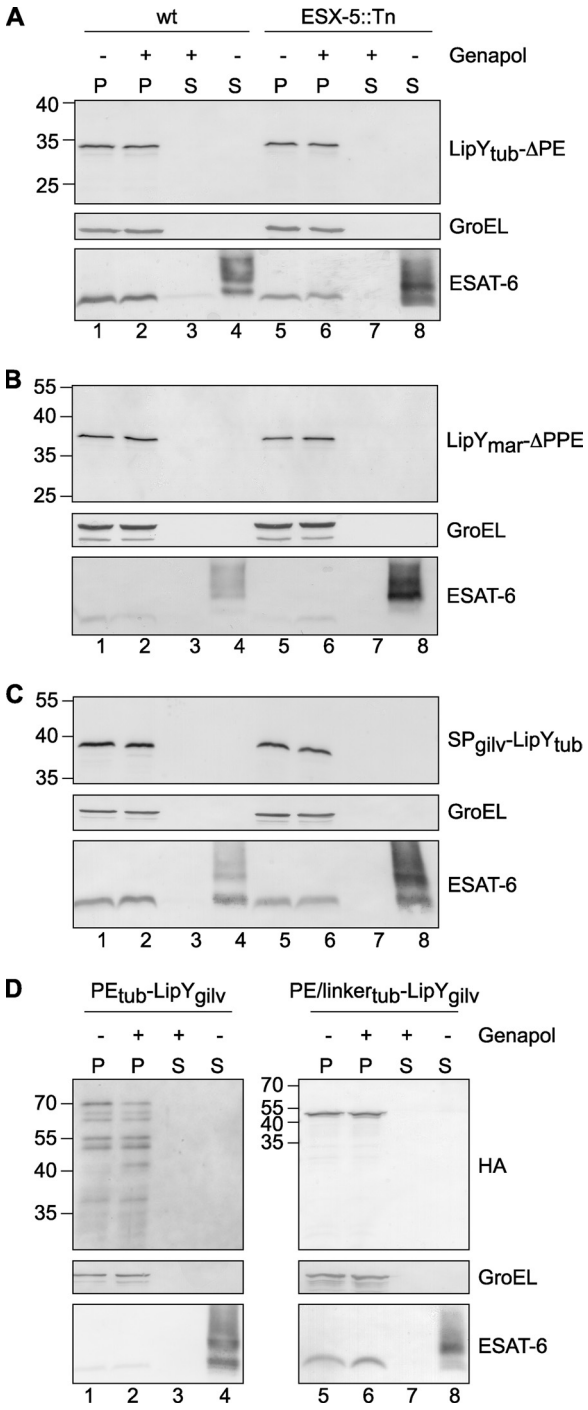

FIGURE 4. **The N-terminal PE and PPE domains are required for ESX-5 secretion of $LipY_{tub}$ and $LipY_{mar}$.** Immunoblot analysis of truncated and chimeric forms of the LipY homologues, expressed in *M. marinum* wt E11 and its ESX-5 mutant 7C1 (*ESX-5::Tn*) (*A–C*) or in E11 alone (*D*), is shown. *A–D*, $LipY_{tub}$ lacking its PE domain ($LipY_{tub}$-ΔPE) (*A*), $LipY_{mar}$ lacking its PPE domain ($LipY_{mar}$-ΔPPE) (*B*), and the chimeras in which the PE domain of $LipY_{tub}$ was replaced by the signal peptide from $LipY_{gilv}$ (SP_{gilv}-$LipY_{tub}$) (*C*) or in which the signal peptide of $LipY_{gilv}$ was replaced by the PE domain of $LipY_{tub}$ (PE_{tub}-$LipY_{gilv}$) or the PE and linker domains of $LipY_{tub}$ were fused to the lipase domain of $LipY_{gilv}$ (PE/$linker_{tub}$-$LipY_{gilv}$) (*D*) were detected with HA antiserum. Equivalent OD units of cell pellets (*P* −), Genapol-treated cells (*P* +), Genapol surface extracts (*S* +), and 2-fold more culture supernatant (*S* −) are shown. GroEL2 and ESAT-6 were detected as controls for cell lysis and secretion, respectively.

the signal peptide encoding sequence (1–45 aa) of $LipY_{gilv}$ (Fig. 1*A*). This chimera was, however, neither secreted nor surface-exposed upon expression in *M. marinum* (Fig. 4*C*, *lanes 3* and *4* and *lanes 7* and *8*). Finally, we also tested whether the PE and PPE domains of $LipY_{tub}$ and $LipY_{mar}$ without the linker and lipase domains could be secreted. To this end, we cloned the PE (1–99 aa) and PPE (1–176 aa) domains of $LipY_{tub}$ and $LipY_{mar}$ and modified them to express C-terminal HA tags (Fig. 1*A*) under the control of the *hsp60* promoter. However, upon introduction into *M. marinum*, neither $PE_{LipYtub}$ nor $PPE_{LipYmar}$ could be detected in immunoblots (results not shown), suggesting that they were not stable. In conclusion, these results show that $LipY_{tub}$ and $LipY_{mar}$ require their respective N-terminal PE and PPE domains for translocation to the cell surface by the ESX-5 secretion machinery.

$LipY_{gilv}$ Fused to a PE Domain Is Not Secreted—To test whether the PE domain can target unrelated proteins for ESX-5 secretion, we replaced the signal peptide of $LipY_{gilv}$ (1–45 aa) with an in-frame N-terminal fusion of the PE domain of $LipY_{tub}$ (1–103 aa) to the linker and lipase domains of $LipY_{gilv}$ (46–494 aa) (Fig. 1*A*). However, upon introduction into *M. marinum*, this chimeric protein was neither surface-exposed nor secreted (Fig. 4*D*, *lanes 3* and *4*). This led us to believe that (part of) the linker domain following the PE domain could be critical. Therefore, a second fusion was constructed in which the signal peptide and the linker domain of $LipY_{gilv}$ (1–236 aa) were replaced by the PE domain and the linker domain of $LipY_{tub}$ (1–205 aa) (Fig. 1*A*). However, this chimera also failed to be secreted (Fig. 4*D*, *lanes 7* and *8*). The first chimeric protein showed the aberrant molecular weight features of the $LipY_{gilv}$ protein, whereas the second construct migrated at the expected molecular weight (Fig. 4*D*, *lanes 1* and *5*), indicating that the linker domain is responsible for the multiple bands observed with $LipY_{gilv}$ in SDS-PAGE, of which some are larger than expected.

These results indicate that heterologous proteins cannot easily be secreted via the ESX-5 pathway. Failure to secrete even a closely related fusion sequence (44% identity on amino acid level) suggests that structure or folding kinetics of substrates may be of importance for ESX-5-dependent translocation.

Processing of Secreted $LipY_{tub}$—As discussed above, secreted and surface-exposed $LipY_{tub}$ is detected in a processed form (Fig. 2*A*). This could mean either that $LipY_{tub}$ is processed during transport or that secreted $LipY_{tub}$ is sensitive to extracellular proteases. To gain more insight into the mechanism of processing, cell lysates of *M. marinum* wild-type strain E11 and its ESX-5 mutant 7C1 overexpressing $LipY_{tub}$ were incubated for 1, 2, 4, and 24 h at 30 °C. No additional processing or degradation of $LipY_{tub}$ was observed in either strain (Fig. 5*A*). These results suggest that LipY is not a protease-sensitive protein, which indicates that processing of $LipY_{tub}$ is a specific maturation event.

Next, processed $LipY_{tub}$ was isolated from the Genapol supernatant (Fig. 5*B*), and the N-terminal sequence was determined by Edman degradation. The results showed that $LipY_{tub}$ is cleaved between Gly-149 and Ala-150 (Fig. 5*C*). The cleavage position indicates that the PE domain and part of the linker domain are removed, whereas the intact lipase domain is retained. The processed protein has a predicted mass of 30.3

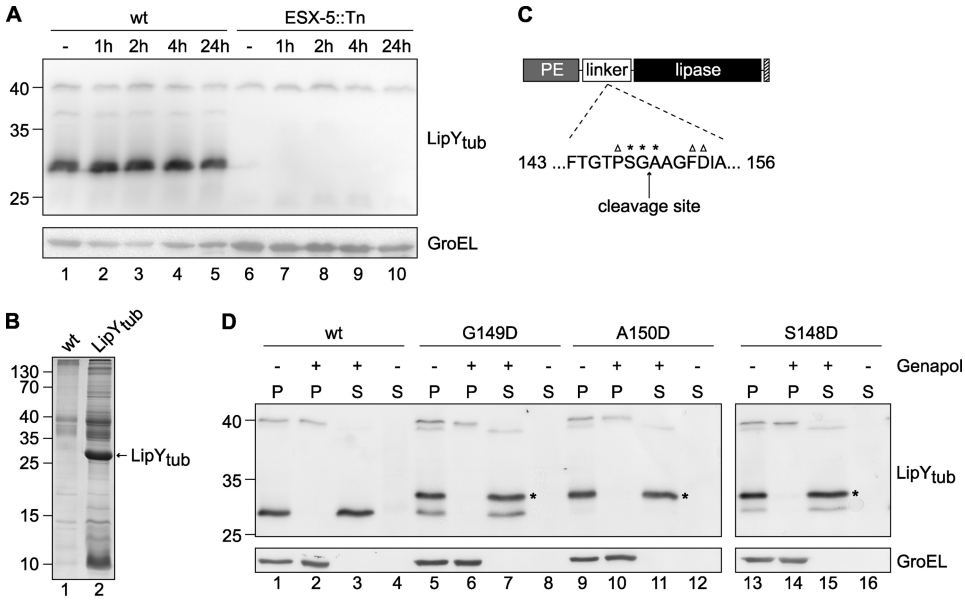

FIGURE 5. **The N terminus is proteolytically removed from secreted LipY$_{tub}$.** *A* and *D*, processing of wt and mutated forms of LipY$_{tub}$ was analyzed by immunoblotting using HA antiserum. GroEL2 was detected as a loading control in *A* and as a control for bacterial lysis in *D*. *A*, no additional processing or degradation was observed when cell lysates of *M. marinum* wt E11 or its ESX-5 mutant 7C1 (*ESX-5::Tn*) expressing LipY$_{tub}$ were incubated for 1, 2, 4, or 24 h after sonication. *B*, Coomassie Brilliant Blue-stained SDS-PAGE gel with Genapol surface extracts of wt *M. marinum* E11 or E11 expressing LipY$_{tub}$. Proteins were transferred to a PVDF membrane, and the band representing processed LipY$_{tub}$ (marked with an *arrow* in the gel) was analyzed by Edman degradation. *C*, LipY$_{tub}$ is processed in the linker domain between residues Gly-149 and Ala-150. Residues altered by site-directed mutagenesis that affected processing are labeled with *asterisks*, whereas those that had no effect are marked with *triangles*. *D*, The S148D, G149D, and A150D point mutations resulted in (partial) production of a surface-exposed slightly larger processed form of LipY$_{tub}$, marked with an *asterisk*, when expressed in *M. marinum* strain M^{vu}. Equal OD units of cell pellets (*P* −), Genapol-treated cell pellet (*P* +), and Genapol surface extract (*S* +), and 2-fold more culture supernatant (*S* −) are shown.

kDa, which correlates well with the observed protein band with an apparent molecular mass of 28 kDa.

To investigate the nature of the recognition sequence of the enzyme responsible for processing of LipY$_{tub}$, we introduced point mutations in residues surrounding the cleavage site (Fig. 5*C*). Most strikingly, substitution of the alanine at position 150 for an aspartic acid residue resulted in a complete disappearance of processed LipY$_{tub}$, whereas a slightly larger cleavage product was visible (Fig. 5*D*, *lane 11*). The S148D and G149D mutations resulted in reduced production of the 28-kDa band and accumulation of a slightly larger product, similar to that resulting from the A150D mutation (Fig. 5*D*, *lanes 7* and *15*). Although the processing was affected, none of these mutations affected the surface localization of LipY$_{tub}$ (Fig. 5*D*, *lanes 7*, *11*, and *15*), nor did they significantly alter the lipase activity (results not shown). Three additional point mutations, P147A, F153A, and D154A, had no effect on LipY$_{tub}$ processing (not shown).

Taken together, these results suggest that the entire PE domain and the first 50 amino acids of the linker domain of LipY$_{tub}$ are removed in a maturation event linked to secretion. Processing depends on the SGA motif encoded by residues 148–150, but if those residues are mutated, cleavage occurs at another adjacent site, and secretion and lipase activity are unaffected.

Expression and Processing of LipY$_{tub}$ in M. tuberculosis and M. bovis BCG—PE and PPE proteins have previously been detected on the surface of *M. bovis* BCG and *M. tuberculosis* (21, 25). To investigate whether LipY$_{tub}$ is also surface-exposed in members of the *M. tuberculosis* complex, we expressed HA-tagged LipY$_{tub}$ in *M. bovis* BCG Copenhagen, *M. bovis* BCG Tice, and the *M. tuberculosis* double auxotroph mutant

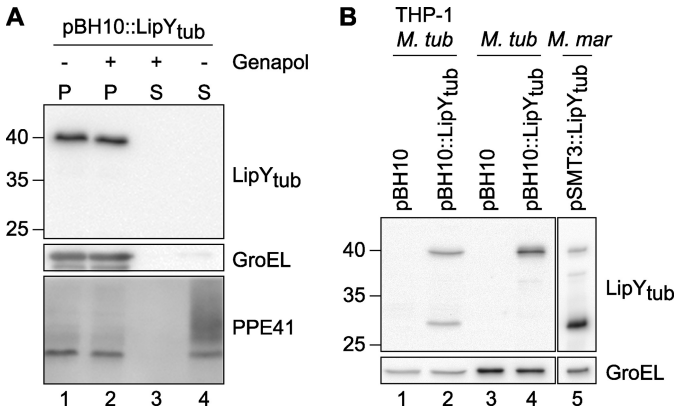

FIGURE 6. **LipY$_{tub}$ processing in *M. tuberculosis* is triggered during infection.** *A*, immunoblot analysis of equal OD units of cell pellet (*P* −), Genapol-treated cell pellet (*P* +), Genapol surface extract (*S* +) fractions, and 2-fold more culture supernatant (*S* −) of a culture of *M. tuberculosis* mc^{2}6020 harboring the pBH10::LipY$_{tub}$ plasmid. *B*, immunoblot showing cell lysates of *M. tuberculosis* (*M. tub*) mc^{2}6020 carrying the empty pBH10 plasmid (*lane 1*) or pBH10::LipY$_{tub}$ (*lane 2*) isolated from THP-1 macrophages at day 2 after infection or from cultures (*lanes 3* and *4*). A cell lysate showing the processing of LipY$_{tub}$ in *M. marinum* (*M. mar*) strain E11 is included in *lane 5*. LipY$_{tub}$ was detected with the HA antibody, GroEL2 was analyzed as control for bacterial lysis in *A* and as a loading control in *B*, and the chromosomally encoded ESX-5 substrate PPE41 was detected as a secretion control in *A*.

mc^{2}6020. Although we observed secretion of endogenous PPE41, a protein that has previously been shown to depend on ESX-5 (3), LipY$_{tub}$ was not was detected in the Genapol-extracted fraction or the culture supernatant (Fig. 6*A*, *lanes 3* and *4*, for results with *M. tuberculosis* mc^{2}6020; data for *M. bovis* BCG strains are not shown). Moreover, unlike in *M. marinum*, LipY$_{tub}$ appeared to remain unprocessed (Fig. 6*A*, *lane 1*). Consistently, no labeling was observed in whole cell immunogold EM (not shown).

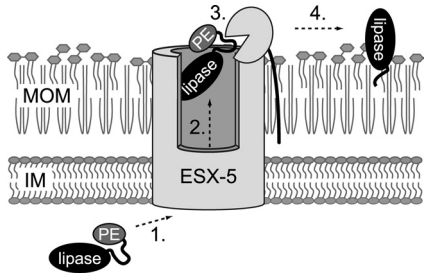

FIGURE 7. **Model of LipY secretion and processing.** Our data support a model in which the PE or PPE domain of the LipY homologues functions as a secretion signal that is recognized by the ESX-5 secretion system (**1**). After recognition, LipY is translocated across the mycobacterial cell envelope by the ESX-5 system (**2**), and during or after translocation the PE or PPE domain is proteolytically removed (**3**), possibly by the mycosin associated with the ESX-5 system. Maturation only occurs in strains with a functional ESX-5 system, and no further processing is observed during extended incubation of lysed bacteria. After secretion, processed LipY is loosely associated with the bacterial surface, where it can hydrolyze TAGs (**4**). *IM*, inner membrane; *MOM*, mycobacterial outer membrane.

Because the expression of *M. tuberculosis lipY* is highly induced upon infection of host cells (46), we hypothesized that the lack of secretion in culture could be due to the fact that additional proteins required for LipY secretion are missing. To test this possibility, we infected human THP-1 cells, differentiated to macrophage-like cells in the presence of phorbol 12-myristate 13-acetate, with *M. tuberculosis* mc^{2}6020 and *M. bovis* BCG Copenhagen expressing HA-tagged $LipY_{tub}$. At 1, 2, 3, and 6 days after infection, the THP-1 cells were lysed by Triton X-100 treatment and centrifuged to collect mycobacteria. Interestingly, these samples showed the same processing pattern for $LipY_{tub}$ as was observed for this protein upon expression in *M. marinum* (see Fig. 6*B*, *lane 2*, for results from day 2 after infection with *M. tuberculosis* mc^{2}6020; data for *M. bovis* BCG are not shown). Together these results indicate that processing of $LipY_{tub}$ occurs both in *M. marinum* and in members of the *M. tuberculosis* complex, albeit indicating that in the latter species, the secretion and/or processing of LipY is induced during infection.

DISCUSSION

Comparative genomics and phylogenetic analyses indicated that the evolution of the type VII secretion system ESX-5 predated the expansion of the PE and PPE gene families in slow-growing mycobacteria (18). These analyses also showed that the recently evolved PE_PGRS and PPE_MPTR (major polymorphic tandem repeat subfamily of PPE proteins) subfamilies have probably originated from PE and PPE genes within the ESX-5 cluster, which resulted in the hypothesis that ESX-5 has a functional link with the recent expansion of PE and PPE proteins. Recently, we showed that ESX-5 is indeed responsible for the secretion of various members of the PE_PGRS and PPE_MPTR protein subfamilies to the cell surface and culture supernatant of *M. marinum* (3, 29). In this work, we report the identification of two novel substrates of the *M. marinum* ESX-5 system, the LipY homologues of *M. tuberculosis* and *M. marinum,* which contain a PE and PPE domain, respectively. Interestingly, the analysis of LipY allows a further expansion of the original hypothesis. Additional *lipY* orthologues were identified in fast-growing mycobacterial species, namely *M. gilvum* and *M. vanbaalenii.* These *lipY* homologues have putative N-terminal signal sequences instead of a PE or PPE domain. $LipY_{gilv}$ was not transported to the cell surface, indicating that the PE or PPE domain is required for surface localization. However, it must be acknowledged that the localization of $LipY_{gilv}$ could not be studied in its native host due to problems with transformation. We would like to propose that LipY homologues in slow-growing mycobacteria have evolved to secreted proteins by substituting the original signal peptide for PE or PPE domains and thereby have turned into substrates of the ESX-5 system (Fig. 7). A remarkable snapshot of this evolution process can be observed in the genome of *M. kansasii,* which has two copies of *lipY*, one containing a PE domain and one with a PPE domain.

N-terminally truncated forms of $LipY_{tub}$ and $LipY_{mar}$ were not transported across the cell envelope, showing that the presence of a PE or PPE domain is required for secretion via ESX-5. Unfortunately, our attempt to investigate whether PE and PPE domains are sufficient for secretion failed because $PE_{LipYtub}$ and $PPE_{LipYmar}$ were unstable when expressed alone. However, in another study, it was shown that the PE domain of PE_PGRS33 can be secreted on its own by ESX-5.[3]

In *M. marinum*, surface-exposed $LipY_{tub}$ and $LipY_{mar}$ were processed, which resulted for $LipY_{tub}$ in the removal of the PE domain and first half of the linker domain. No further processing or degradation was observed of $LipY_{tub}$ after extended incubation of *M. marinum* lysates, indicating that LipY cleavage results from a specific maturation process, linked to ESX-5 translocation (Fig. 7). Similar patterns of N-terminal processing have previously been observed for PE_PGRS and PPE_MPTR proteins secreted by ESX-5 (29), although the responsible protease was not identified. This could mean that processing of ESX-5 substrates is a common process, similar to the removal of a signal sequence. Interestingly, processing has also been observed for the ESX-1 substrate EspB (16), which was recently shown to be cleaved by the ESX-1-encoded protease MycP1 (47). To further investigate the nature of the $LipY_{tub}$ cleavage site, we altered the neighboring residues using site-directed mutagenesis. Although introducing point mutations could block processing at the original cleavage site, the mutations always resulted in an alternative processing pattern, as is also observed for signal sequence removal (48, 49). Besides the removal of the secretion domain, processing could also have a functional implication. *M. tuberculosis* utilizes fatty acids as the principal energy source during the latent stage of infection (50–52), and it has been shown that it stores fatty acids in the form of TAGs (51). Dormancy leads to high up-regulation of $LipY_{tub}$, whereas a $\Delta lipY_{tub}$ mutant has a severely compromised ability to degrade stored TAGs upon starvation (32). This indicates that LipY is involved in degradation of intracellular TAGs. In this study, we show that surface-exposed $LipY_{tub}$ can also hydrolyze extracellular lipids. LipY may therefore also be involved in fatty acid acquisition by hydrolyzing host lipids during infection. Mishra *et al.* (33) showed that the lipase activity of

[3] A. Cascioferro, M. H. Daleke, W. Bitter, and R. Manganelli, manuscript in preparation.

both $LipY_{tub}$ and $LipY_{mar}$ is significantly higher in the absence of the PE or PPE domains. Therefore, processing of surface-exposed LipY will result in increased lipase activity and more efficient hydrolysis of host lipids.

Intriguingly, $LipY_{tub}$ was found to be neither surface-exposed nor processed when expressed in cultures of *M. tuberculosis* and *M. bovis* BCG. However, infection of THP-1 macrophages with *M. tuberculosis* or *M. bovis* BCG did result in $LipY_{tub}$ processing, suggesting that the pathway required for secretion and/or processing in *M. tuberculosis* complex members is activated upon infection of host cells. Endogenous *M. tuberculosis lipY* is induced upon infection of macrophages (46), and it is conceivable that additional proteins required for LipY secretion and processing are also up-regulated during an infection. These proteins are not the ESX-5 system itself, which seems to be constitutively produced. Instead, it could for instance be a specific chaperone required for secretion or because proteins secreted via type VII secretion systems are often secreted as dimers, another ESX-5 substrate (3, 53).

Our data showing that the PE/PPE domain of LipY is essential for secretion conflict with the results presented in Ref. 33, where it was shown that localization of $LipY_{tub}$ and $LipY_{mar}$ in the cell wall of *M. smegmatis* and *M. bovis* BCG and on the bacterial surface of *M. bovis* BCG is independent of these domains. Moreover, our analysis in *M. tuberculosis* and *M. bovis* BCG showed that LipY is not surface-exposed when grown in culture. Although we cannot explain these differences, we have analyzed the LipY secretion pathway in more detail using ESX-5 mutants. Immunoblotting and whole cell immunogold labeling EM showed a clear lack of surface localization of both $LipY_{tub}$ and $LipY_{mar}$ in the ESX-5 mutant, demonstrating that these are indeed ESX-5 substrates. This was again confirmed in our whole cell lipase assay, in which the *p*-nitrophenyl stearate hydrolysis observed with $LipY_{tub}$ in wild-type *M. marinum* was absent in the ESX-5 mutant strain.

Some PE and PPE proteins are encoded in operons (18). One such couple, consisting of PE25 and PPE41, forms a complex (54) and is secreted by ESX-5 as a heterodimer.[4] However, computational predictions suggest that many other PE and PPE proteins, including several that are encoded alone in the genome, also have cognate interaction partners (55). This raises the hypothesis that PE and PPE proteins are targeted to ESX-5 in pairs. Experiments are underway in our laboratory to investigate how PE and PPE domains may interact in targeting to ESX-5 and to identify the residues within the PE and PPE domains that constitute the ESX-5 secretion signal.

Acknowledgments—We are grateful to Edith Houben and Ben Appelmelk for helpful discussions and to Aniek van der Woude and Eveline Weerdenburg for technical assistance. We also thank Ida Rosenkrands for kindly providing the ESAT-6 antibody.

REFERENCES

1. Brennan, P. J., and Nikaido, H. (1995) *Annu. Rev. Biochem.* **64,** 29–63
2. Abdallah, A. M., Gey van Pittius, N. C., Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., and Bitter, W. (2007) *Nat. Rev. Microbiol.* **5,** 883–891
3. Abdallah, A. M., Verboom, T., Hannes, F., Safi, M., Strong, M., Eisenberg, D., Musters, R. J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., Luirink, J., and Bitter, W. (2006) *Mol. Microbiol.* **62,** 667–679
4. DiGiuseppe Champion, P. A., and Cox, J. S. (2007) *Cell. Microbiol.* **9,** 1376–1384
5. Gao, L. Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J. N., and Brown, E. J. (2004) *Mol. Microbiol.* **53,** 1677–1693
6. Guinn, K. M., Hickey, M. J., Mathur, S. K., Zakel, K. L., Grotzke, J. E., Lewinsohn, D. M., Smith, S., and Sherman, D. R. (2004) *Mol. Microbiol.* **51,** 359–370
7. Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K. E., Marchal, G., Leclerc, C., and Cole, S. T. (2003) *Nat. Med.* **9,** 533–539
8. Stanley, S. A., Raghavan, S., Hwang, W. W., and Cox, J. S. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 13001–13006
9. Gey Van Pittius, N. C., Gamieldien, J., Hide, W., Brown, G. D., Siezen, R. J., and Beyers, A. D. (2001) *Genome Biol.* **2,** RESEARCH0044
10. Tekaia, F., Gordon, S. V., Garnier, T., Brosch, R., Barrell, B. G., and Cole, S. T. (1999) *Tuber. Lung Dis.* **79,** 329–342
11. Brodin, P., Rosenkrands, I., Andersen, P., Cole, S. T., and Brosch, R. (2004) *Trends Microbiol.* **12,** 500–508
12. Raghavan, S., Manzanillo, P., Chan, K., Dovey, C., and Cox, J. S. (2008) *Nature* **454,** 717–721
13. Carlsson, F., Joshi, S. A., Rangell, L., and Brown, E. J. (2009) *PLoS Pathog.* **5,** e1000285
14. Fortune, S. M., Jaeger, A., Sarracino, D. A., Chase, M. R., Sassetti, C. M., Sherman, D. R., Bloom, B. R., and Rubin, E. J. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 10676–10681
15. MacGurn, J. A., Raghavan, S., Stanley, S. A., and Cox, J. S. (2005) *Mol. Microbiol.* **57,** 1653–1663
16. McLaughlin, B., Chon, J. S., MacGurn, J. A., Carlsson, F., Cheng, T. L., Cox, J. S., and Brown, E. J. (2007) *PLoS Pathog.* **3,** e105
17. Sani, M., Houben, E. N., Geurtsen, J., Pierson, J., de Punder, K., van Zon, M., Wever, B., Piersma, S. R., Jiménez, C. R., Daffé, M., Appelmelk, B. J., Bitter, W., van der Wel, N., and Peters, P. J. (2010) *PLoS Pathog.* **6,** e1000794
18. Gey van Pittius, N. C., Sampson, S. L., Lee, H., Kim, Y., van Helden, P. D., and Warren, R. M. (2006) *BMC Evol. Biol.* **6,** 95
19. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393,** 537–544
20. Basu, S., Pathak, S. K., Banerjee, A., Pathak, S., Bhattacharyya, A., Yang, Z., Talarico, S., Kundu, M., and Basu, J. (2007) *J. Biol. Chem.* **282,** 1039–1050
21. Brennan, M. J., Delogu, G., Chen, Y., Bardarov, S., Kriakov, J., Alavi, M., and Jacobs, W. R., Jr. (2001) *Infect. Immun.* **69,** 7326–7333
22. Li, Y., Miltner, E., Wu, M., Petrofsky, M., and Bermudez, L. E. (2005) *Cell. Microbiol.* **7,** 539–548
23. Nair, S., Ramaswamy, P. A., Ghosh, S., Joshi, D. C., Pathak, N., Siddiqui, I., Sharma, P., Hasnain, S. E., Mande, S. C., and Mukhopadhyay, S. (2009) *J. Immunol.* **183,** 6269–6281
24. Ramakrishnan, L., Federspiel, N. A., and Falkow, S. (2000) *Science* **288,** 1436–1439
25. Banu, S., Honoré, N., Saint-Joanis, B., Philpott, D., Prévost, M. C., and Cole, S. T. (2002) *Mol. Microbiol.* **44,** 9–19
26. Cascioferro, A., Delogu, G., Colone, M., Sali, M., Stringaro, A., Arancia, G., Fadda, G., Palù, G., and Manganelli, R. (2007) *Mol. Microbiol.* **66,** 1536–1547
27. Delogu, G., Pusceddu, C., Bua, A., Fadda, G., Brennan, M. J., and Zanetti, S. (2004) *Mol. Microbiol.* **52,** 725–733
28. Sampson, S. L., Lukey, P., Warren, R. M., van Helden, P. D., Richardson, M., and Everett, M. J. (2001) *Tuberculosis* **81,** 305–317

[4] M. H. Daleke, R. Ummels, C. M. Vandenbroucke-Grauls, J. Luirink, and W. Bitter, manuscript in preparation.

29. Abdallah, A. M., Verboom, T., Weerdenburg, E. M., Gey van Pittius, N. C., Mahasha, P. W., Jiménez, C., Parra, M., Cadieux, N., Brennan, M. J., Appelmelk, B. J., and Bitter, W. (2009) *Mol. Microbiol.* **73,** 329–340
30. Hermans, P. W., van Soolingen, D., and van Embden, J. D. (1992) *J. Bacteriol.* **174,** 4157–4165
31. Poulet, S., and Cole, S. T. (1995) *Arch. Microbio.l* **163,** 87–95
32. Deb, C., Daniel, J., Sirakova, T. D., Abomoelak, B., Dubey, V. S., and Kolattukudy, P. E. (2006) *J. Biol. Chem.* **281,** 3866–3875
33. Mishra, K. C., de Chastellier, C., Narayana, Y., Bifani, P., Brown, A. K., Besra, G. S., Katoch, V. M., Joshi, B., Balaji, K. N., and Kremer, L. (2008) *Infect. Immun.* **76,** 127–140
34. Puttinaowarat, S., Thompson, K. D., and Adams, A. (2000) *Fish Vet. J.* **5,** 6–21
35. Ramakrishnan, L., and Falkow, S. (1994) *Infect. Immun.* **62,** 3222–3229
36. Dean-Ross, D., and Cerniglia, C. E. (1996) *Appl. Microbiol. Biotechnol.* **46,** 307–312
37. Behr, M. A., Wilson, M. A., Gill, W. P., Salamon, H., Schoolnik, G. K., Rane, S., and Small, P. M. (1999) *Science* **284,** 1520–1523
38. Leung, A. S., Tran, V., Wu, Z., Yu, X., Alexander, D. C., Gao, G. F., Zhu, B., and Liu, J. (2008) *BMC Genomics* **9,** 413
39. Sampson, S. L., Dascher, C. C., Sambandamurthy, V. K., Russell, R. G., Jacobs, W. R., Jr., Bloom, B. R., and Hondalus, M. K. (2004) *Infect. Immun.* **72,** 3031–3037
40. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R., Jr. (1990) *Mol. Microbiol.* **4,** 1911–1919
41. Stolt, P., and Stoker, N. G. (1996) *Microbiology* **142,** 2795–2802
42. Hayward, C. M., O'Gaora, P., Young, D. B., Griffin, G. E., Thole, J., Hirst, T. R., Castello-Branco, L. R., and Lewis, D. J. (1999) *Vaccine* **17,** 1272–1281
43. Bachrach, G., Colston, M. J., Bercovier, H., Bar-Nir, D., Anderson, C., and Papavinasasundaram, K. G. (2000) *Microbiology* **146,** 297–303
44. Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G., and Parkhill, J. (2005) *Bioinformatics* **21,** 3422–3423
45. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) *Mol. Biol. Evol.* **24,** 1596–1599
46. Srivastava, V., Rouanet, C., Srivastava, R., Ramalingam, B., Locht, C., and Srivastava, B. S. (2007) *Microbiology* **153,** 659–666
47. Ohol, Y. M., Goetz, D. H., Chan, K., Shiloh, M. U., Craik, C. S., and Cox, J. S. (2010) *Cell Host Microbe* **7,** 210–220
48. Ghrayeb, J., Lunn, C. A., Inouye, S., and Inouye, M. (1985) *J. Biol. Chem.* **260,** 10961–10965
49. Suominen, I., Meyer, P., Tilgmann, C., Glumoff, T., Glumoff, V., Käpylä, J., and Mäntsälä, P. (1995) *Microbiology* **141,** 649–654
50. Bloch, H., and Segal, W. (1956) *J. Bacteriol.* **72,** 132–141
51. Daniel, J., Deb, C., Dubey, V. S., Sirakova, T. D., Abomoelak, B., Morbidoni, H. R., and Kolattukudy, P. E. (2004) *J. Bacteriol.* **186,** 5017–5030
52. Muñoz-Elías, E. J., and McKinney, J. D. (2005) *Nat. Med.* **11,** 638–644
53. DiGiuseppe Champion, P. A., Stanley, S. A., Champion, M. M., Brown, E. J., and Cox, J. S. (2006) *Science* **313,** 1632–1636
54. Strong, M., Sawaya, M. R., Wang, S., Phillips, M., Cascio, D., and Eisenberg, D. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 8060–8065
55. Riley, R., Pellegrini, M., and Eisenberg, D. (2008) *PLoS Comput. Biol.* **4,** e1000174